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Purification and characterization of a monoclonal T-cell suppressor factor specific for poly(LGlu^{60}LAla^{30}LTyr^{10})

(T-cell hybridoma/antigen-binding peptide/isolation)

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ABSTRACT **A monoclonal T cell-derived suppressor factor specific for the terpolymer poly(LGlu^{60}LAla^{30}LTyr^{10}) produced by the T-cell hybridoma 258 C4.4, was purified to homogeneity. This was accomplished by fractionation of the culture medium by using a combination of affinity chromatography and reverse-phase and ion-exchange high-performance liquid chromatography. The purified factor is composed of a single M_r 24,000 polypeptide chain, and the homogeneous protein maintains the ability to suppress antibody and T-cell proliferative responses to poly(LGlu^{60}LAla^{30}LTyr^{10}) specifically. The specific activity of pure suppressor factor is calculated to be 8×10^7 units/μg.**

The immune response to the synthetic terpolymer poly(LGlu^{60}LAla^{30}LTyr^{10}) (GAT) by inbred strains of mice is controlled by an immune response (*Ir* gene) that maps to the *K*, *I-A* subregions of the *H-2* gene complex (1, 2). Immunization with GAT stimulates antibody formation *in vivo* and *in vitro* and primes for T-cell proliferation to GAT *in vitro* in lymph node cells from mice bearing the responder *H-2*^{a,b,d,f,k} haplotypes (3, 4). In nonresponder (*H-2*^{p,q,s}) mice, immunization with GAT does not stimulate GAT-specific plaque-forming-cell (PFC) or T-cell proliferative responses, unless GAT is complexed with a carrier such as methylated bovine serum albumin (GAT–methylated albumin) (2–4). To date, our studies suggest that lack of responses to GAT in nonresponder mice is correlated with development of GAT-specific suppressor T cells (5). Extracts of GAT-specific suppressor T cells from nonresponder mice contain a soluble suppressor factor(s), called GAT-TsF, that inhibits PFC responses to GAT–methylated albumin in nonresponder mice *in vivo* and *in vitro* (6, 7) and inhibits T-cell proliferative responses by nonresponder mice primed with GAT–methylated albumin (8). Affinity-purified GAT-TsF is a protein of estimated M_r 45,000–60,000 determined by gel filtration and has the ability to bind specifically to GAT (9). The antigen-binding moiety bears determinants that appear to be encoded both by the Ig variable heavy chain gene complex and the *I-J* subregion of the major histocompatibility gene complex (10, 11).

It has not been possible to purify GAT-TsF to homogeneity because of the extremely small quantity of active material in these extracts. Therefore, we have constructed T-cell hybridomas by fusing the AKR thymoma BW5147 and splenic T cells from GAT-primed nonresponder DBA/1 mice (12). Hybridomas were screened by bioassay for constitutive synthesis of GAT-TsF, and selected hybridomas were cloned. The medium from the hybridomas provides a rich source of GAT-TsF; each milliliter of medium contains 20–40 times the amount of suppressive material that can be obtained in an extract of all of the lymphoid cells from an entire mouse. In addition, protein purification and detection methodologies have been developed to the point that detection of picomolar quantities of protein may be considered routine (13, 14).

In this communication, we report the successful purification of the monoclonal T-cell product GAT-TsF to homogeneity by use of affinity chromatography and high performance liquid chromatography (HPLC). To monitor the purification of this protein, the biological activity was determined by specific suppression of PFC or T-cell proliferative responses, or both, at each step.

MATERIALS AND METHODS

Mice. DBA/1 and B10.Q (*H-2*^{q}) mice were bred in the Animal Resources facility at the Jewish Hospital of St. Louis and were 3–6 mo old when used. *H-2*^{q} mice are nonresponders to GAT.

Antigen and Immunization. GAT was purchased from Vega–Fox (Tucson, AZ); methylated bovine serum albumin was purchased from Sigma. The insoluble complex, GAT–methylated albumin, was prepared as described (3). DBA/1 or B10.Q mice were injected in the hind foot pad with 5–20 μg of GAT as GAT–methylated albumin emulsified in complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37 Ra (8).

T-Cell Hybridoma Lines. Splenic T cells (enriched by passage through nylon wool) from GAT-primed, nonresponder DBA/1 mice were fused to the hypoxanthine/guanine phosphoribosyltransferase-deficient AKR thymoma BW5147 by the technique of Galfre *et al.* (15). Clones producing GAT-TsF constitutively were isolated and have been maintained in tissue culture or kept frozen in liquid nitrogen. GAT-TsF from the prototype hybridoma 258 C4.4 has been characterized extensively, and was used in the studies reported here.

Cell Culture and Assay of Hybridoma T-Cell Supernates. Development of primary splenic PFC responses was measured *in vitro* under modified Mishell–Dutton conditions as described (5–7). PFC responses in cultures stimulated with GAT–methylated albumin or sheep erythrocytes were assayed on day 5 with GAT–sheep erythrocyte complexes or sheep erythrocytes as indicator cells. T-cell proliferative responses to GAT by lymph node cells from mice immunized with GAT–methylated albumin were measured as described (4). The relative suppressive activity was determined by titration, and the specificity

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Abbreviations: GAT, poly(LGlu^{60}LAla^{30}LTyr^{10}); PFC, plaque-forming cell(s); HPLC, high-performance liquid chromatography; S_{50}, 50% suppression of the response; GAT–methylated albumin, GAT complexed with methylated bovine serum albumin; GAT-TsF, soluble suppressor factor(s) from GAT-specific suppressor T cells.

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was verified by addition of GAT-TsF to cultures stimulated with an irrelevant antigen. The proliferation assay and the antibody-forming-cell assay give linear titration curves over the dilutions tested (e.g., 10^{-3}–10^{-9}), allowing direct estimation of relative potency (6, 12). Supernatants from the parent thymoma BW5147 and control T-cell hybridomas contained no detectable GAT-TsF at dilutions of 1:1000 (12). The data have been expressed as the inverse of the final dilution of supernate or purified factor that causes 50% suppression of the response (S_{50} units/ml). Titration of suppressive activity was carried out at dilutions ranging from 5×10^{-3} to 1×10^{-9}. As the material was purified, lower dilutions were eliminated. Thus, small amounts of biological activity (e.g., less than 1000 S_{50} units) would not be detected.

Preparation and Use of Immunosorbents. To remove cell debris and serum proteins that absorb nonspecifically to antigen-coupled Sepharose, culture supernatants were prefiltered through bovine serum albumin-Sepharose at 4°C. The effluent, containing GAT-TsF, was collected and applied to GAT-Sepharose, which was washed extensively and subsequently eluted with 2.0 M KCl (4).

HPLC. Affinity-purified GAT-TsF was applied to a column containing Lichrosorb RP-8 (E. Merck, Darmstadt, Federal Republic of Germany) in a reverse-phase buffer of 0.5 M acetic acid/1.0 M pyridine, pH 5.5. The absorbed proteins were eluted with a stepwise gradient of 0–50% 1-propanol (16). An automated fluorescent detection system using fluorescamine (Hoffmann–La Roche) was used for monitoring protein in the column effluent (17). Ion-exchange HPLC was carried out on a column of 10-μm DEAE-Nugel DE-200 (Separation Industries, Orange, NJ). Proteins were eluted from DEAE columns with a linear gradient from 0.1 M acetic acid/0.2 M pyridine to 3 M acetic acid/6 M pyridine at a flow rate of 13 ml/hr.

Dansylation of GAT-TsF. Proteins were labeled with [^{3}H]dansyl chloride by lyophilizing the sample and reconstituting it in 50 μl of 0.1 M lithium borate. [^{3}H]Dansyl chloride (specific activity, 13.7 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Amersham-Searle) was lyophilized and reconstituted in acetone. Proteins were added to 1.1 nmol of [^{3}H]dansyl chloride and allowed to react for 1 hr. The reaction was stopped by the addition of ammonium bicarbonate (0.1 M; 10 μl). Bovine serum albumin (25 μg) was added to each sample as the carrier protein. For $NaDodSO_4$/polyacrylamide gel electrophoresis, the labeled samples were lyophilized and resuspended in a reducing buffer of 0.09 M Tris·HCl, pH 6.8/1.0% $NaDodSO_4$/5.0% 2-mercaptoethanol/10% glycerol/7 M urea/0.001% bromophenol blue (18).

Gel Electrophoresis. Purified, tritiated samples or unlabeled samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing or nonreducing conditions in 15% polyacrylamide gels (18). The gels that contained radioactive samples were fixed either in methanol/acetic acid/water, 10:7:83 (vol/vol), or in EN^{3}HANCE (New England Nuclear). The gels were dried and placed against Kodak x-ray film (XAR-5) for autoradiography.

Amino Acid Analysis. Samples were dried *in vacuo* and hydrolyzed in 6 M HCl/0.1% thioglycollic acid at 110°C for 24 hr. After drying, the samples were dissolved in pH 2.2 buffer and analyzed in a fluorescamine amino acid analyzer as described (19).

RESULTS

Purification of GAT-TsF. Six liters of supernatant fluid (in 2-liter portions) from cultures of C4.4, containing 20,000 S_{50} units/ml, were prefiltered over a bovine serum albumin-Sepharose column and applied to a GAT-Sepharose column, and GAT-TsF was eluted with 2.0 M KCl (16). The KCl eluate was subsequently applied to the Lichrosorb RP-8 column and eluted with a stepwise gradient of 1-propanol in a pyridine/acetate buffer. A sample from each fraction was assayed for GAT-specific suppressive activity in PFC or T-cell proliferative responses, or both, *in vitro* (Fig. 1*A*). Specific suppressive activity was detected only in two fractions eluting in 45% propanol. Reverse-phase HPLC of each two-liter fraction produced a protein profile similar to that in Fig. 1*A*, and the biologically active material was identified in the same two fractions. It is clear from the protein profile in Fig. 1*A*, that the majority of the protein obtained from the GAT-Sepharose column was extraneous protein. This is most likely due to the negative charge of GAT-Sepharose, causing it to behave as an ion-exchange resin and as a specific adsorbent.

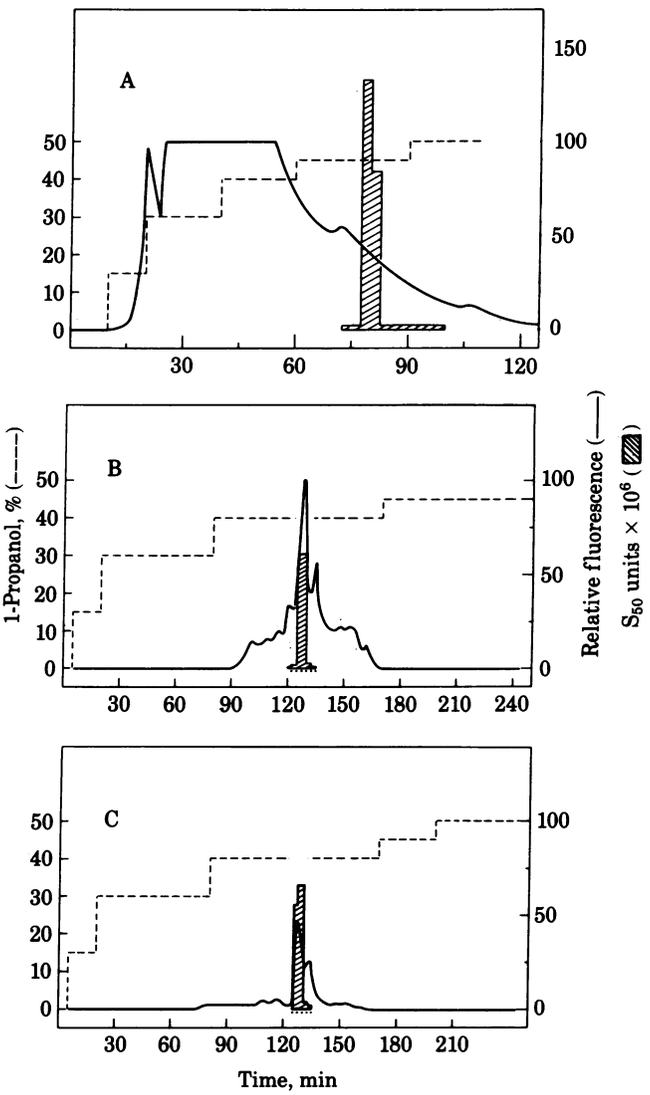

FIG. 1. Purification of GAT-TsF by reverse-phase HPLC. Supernatant fluid was purified by affinity chromatography and applied to a Lichrosorb RP-8 10-μm column. The adsorbed material was eluted, and a portion (6%) of the effluent was directed to the fluorescamine detection system. (*A*) Affinity-purified GAT-TsF from 2 liters of supernatant was fractionated on a 250 × 10 mm preparative column at a fast flow rate (160 ml/hr). (*B*) Bioactive fractions from *A* were pooled with the active fractions from two additional batches, diluted, and rechromatographed on a 250 × 4.6 mm analytical column at a slow flow rate (13 ml/hr). (*C*) Fractions between 125 and 135 min were pooled, diluted, and rechromatographed by following the same procedure as in *B*.

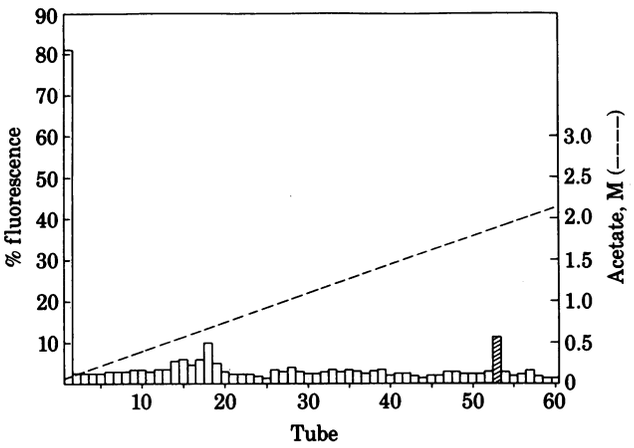

FIG. 2. DEAE ion-exchange HPLC of GAT-TsF purified by reverse-phase HPLC. The bioactive material from *C* in Fig. 1 was applied to the column of 10-μm DEAE Nugel DE-200. The adsorbed protein was eluted with a linear gradient from 0.1 M acetic acid/0.2 M pyridine to 3 M acetic acid/6 M pyridine at a flow rate of 13 ml/hr. Protein from each fraction was measured by bypass fluorescamine analysis. No bioactivity or protein was detectable in fractions eluting between 2.0 and 3.0 M acetate.

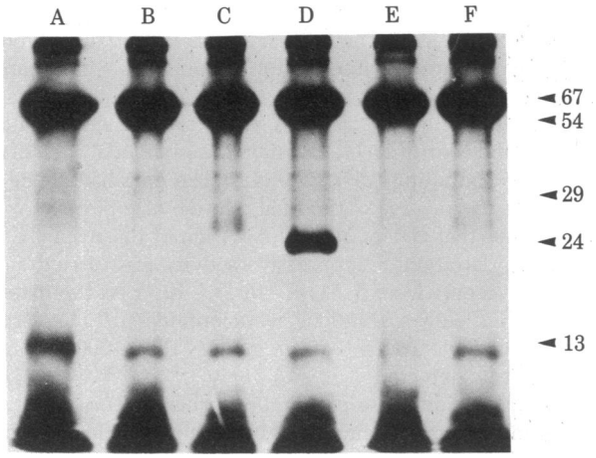

FIG. 3. $NaDodSO_4$ polyacrylamide gel electrophoresis of GAT-TsF purified by DEAE ion-exchange HPLC. Lanes: A–C, fractions 1, 3, and 17, respectively, from DEAE chromatography (Fig. 2); D, fraction 53 (the bioactive fraction); E and F, fractions 54 and 55 (which had no bioactivity). All samples were lyophilized and labeled with [^{3}H]dansyl chloride. The reaction was stopped with ammonium bicarbonate and bovine serum albumin. M_rs are shown $\times 10^{-3}$.

The tubes containing the maximum suppressive activity that eluted in 45% propanol were pooled and rechromatographed on an RP-8 column with a shallower propanol gradient and a slower flow rate. A broad band of protein was eluted by 40% propanol, but the only detectable biologically active material was confined to two fractions (Fig. 1*B*). When these two fractions were pooled and rechromatographed over the RP-8 column at a slow flow rate, the bioactive protein eluted at precisely the same time as in the previous run (Fig. 1*C*). Finally, material from the two fractions containing biologically active material from the fractionation shown in Fig. 1*C* was applied to a DEAE ion-exchange HPLC column. The absorbed material was eluted with an acetate gradient (from 0.1 M acetic acid/0.2 M pyridine to 3.0 M acetic acid/6.0 M pyridine), and each fraction was assayed for protein and biological activity (Fig. 2). The material applied to the DEAE column was resolved into two peaks (tubes 18 and 53); however, the biological activity was confined to one protein fraction (tube 53).

A summary of the purification of GAT-TsF from 6 liters of supernatant from clone C4.4 is shown in Table 1. Six liters of crude supernate contained 4×10^7 S_{50} units of GAT-TsF; after affinity purification, the 6 liters contained 10^8 S_{50} units. This apparent 2- to 3-fold enrichment in suppressive activity has been routinely observed (12) and is most likely due to the fact that these culture supernatants contain nonspecific enhancing materials that compete with GAT-TsF, thereby causing an underestimate of the original suppressive activity in the crude supernatant fluid. By the last step in the purification, 1.4×10^8 S_{50} units were contained in fraction 53 from the HPLC DEAE-column separation. This purification scheme has been performed on two large preparations (6 and 10 liters) with identical results. The major advantages of HPLC are its reproducibility and the sharp resolution of individual peaks. This is illustrated by the fact that the DEAE-Nugel chromatography step shows all the bioactivity eluting at a single time point in several runs. This is true not only for TsF isolated from C4.4 but also for a second GAT-TsF (*H-2*^{s} haplotype), which also elutes in a single fraction on DEAE-Nugel (unpublished data).

M_r Estimated by Polyacrylamide Gel Electrophoresis. Fractions 1, 3, 17, 53, 54, and 55 from the DEAE ion-exchange HPLC chromatography were dansylated and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions. A single band with an estimated M_r of 24,000 was present in the bioactive fraction (Fig. 3, lane D). The dark band at the top of each lane in Fig. 3 is the carrier bovine serum albumin (M_r 67,000). The addition of the albumin was necessary

Table 1. Summary of purification of GAT-TsF

Steps	Total protein,* ng	Bioactivity,† S_{50} units $\times 10^7$	Degree of purity, S_{50} units/ng	Purification factor
Crude supernatant	2.6×10^{10}	4.0	1.5×10^{-3}	1
Albumin-Sepharose effluent	2.2×10^{10}	8.0	3.7×10^{-3}	2.5
GAT-Sepharose eluate	1.1×10^8	10	9.5×10^{-1}	6.3×10^2
HPLC (*A*)‡	1.0×10^5	36	3.6×10^3	2.4×10^6
HPLC (*B*)	2.0×10^4	12	6.1×10^3	4.1×10^6
HPLC (*C*)	4.4×10^3	12	2.7×10^4	1.8×10^7
DEAE-HPLC	1.8×10^3	14	7.8×10^4	5.2×10^7

* Protein concentration was measured either directly by bypass analysis or estimated from a portion of the column eluate directed through the fluorescence detection system. Concentration of protein (versus a bovine serum albumin calibration standard) can be determined in the low picomole or nanogram level.

† Bioactivity was measured by inhibition of the GAT-stimulated PFC or T-cell proliferative responses; no inhibition of responses to irrevelant control antigens was detected.

‡ Letters in parentheses correspond to panels in Fig. 1.

Table 2. Amino acid composition of GAT-TsF

Amino acid	GAT-TsF, pmol*			
	Sample 1	Sample 2	Average	Residues†
Asp	511	404	458	18.0
Thr	276	243	260	10.2
Ser	405	ND	405	15.8
Glu	935	ND	935	36.6
Gly	737	570	654	25.6
Ala	373	348	361	14.2
Val	288	277	283	11.0
Met	59	43	51	2.0
Ile	125	125	125	5.0
Leu	358	355	357	14.0
Tyr	205	199	202	8.0
Phe	155	177	166	6.6
His	124	126	125	5.0
Lys	388	362	275	14.8
Arg	336	ND	336	13.2
			Total	200

ND, Not determined.

* Purified biologically active GAT-TsF (0.6 μg) was hydrolyzed by HCl. A column buffer sample from a blank run was analyzed and subtracted from each protein sample. The levels of proline, tryptophan, and cysteine were not determined.

† The number of residues was determined by assuming that methionine was present in the lowest molar amount. In order to arrive at a M_r by amino acid analysis, the number of residues of methionine was set at two per molecule. This gave a total M_r of 20,000 by amino acid content. If one allows for the presence of carbohydrate and incomplete amino acid analysis, this is close to the M_r value obtained by polyacrylamide gel analysis.

to recover the dansylated TsF because of difficulties encountered in applying the material to the gel. This presumably is caused by increased hydrophobicity of the dansylated protein, causing it to lose solubility in aqueous media. Other bands represent reaction products of dansyl chloride and are present in all lanes, even those containing dansyl chloride and buffer but no detectable protein or bioactivity (for example, Fig. 3, lanes E and F). The M_r 24,000 peptide is also observed at the same mobility under nonreducing conditions (not shown). Similarly, a single peptide band at M_r 24,000 was observed with a gradient gel system (refs. 20 and 21; data not shown). To verify that the M_r 24,000 protein was GAT-TsF, a 15% polyacrylamide gel electrophoresis was performed with unlabeled material from fraction 53 of the DEAE ion-exchange HPLC. Electrophoretic analysis showed $\approx 6 \times 10^6$ S_{50} units of biological activity. The gel was cut into 3-mm slices, and each slice was homogenized in a phosphate buffer containing 1 mg of bovine serum albumin. These extracts were analyzed for bioactivity. A single bioactive fraction containing 1×10^7 S_{50} units (100% recovery), with an estimated M_r of 24,000, was detected.

Amino Acid Analysis. Amino acid analysis of homogeneous GAT-TsF was performed with the fluorescamine amino acid analyzer on 0.6-μg samples of GAT-TsF. The amino acid analysis is summarized in Table 2. The analysis was performed on two lots of purified GAT-TsF and on the appropriate sample taken from a blank chromatography run (i.e., a chromatography run performed without any added protein). The amino acid compositions obtained in the two runs were comparable.

DISCUSSION

Based on the fact that we have isolated a protein that has antigen-specific suppressive activity and that the protein and biological activity copurify as a single moiety by using two different physical separation methods (hydrophobicity or charge), we conclude that GAT-TsF has been purified to homogeneity. This was achieved by a combination of affinity chromatography and reverse-phase HPLC. The reverse-phase and DEAE ion-exchange HPLC steps resulted in a final purification factor of 5.2×10^7. From 6000 ml of culture supernate, ≈ 2 μg of purified protein was obtained. Because only fractions containing the peak suppressive activity were selected at each step of the purification scheme, this recovery could be as low as 50% of the bioactive material. Therefore, the original supernatant fluids may contain up to 0.5–1.0 ng of GAT-TsF per ml. If the specific activity (7.8×10^4 S_{50} units/ng) of this hybridoma product is a close approximation of the specific activity of factors produced *in vivo*, then extracts of the lymphoid cells from GAT-primed mice would yield about 0.013 ng of GAT-TsF per mouse.

Based on the polyacrylamide gel analysis by staining and bioactivity, the purified protein appears to be a single M_r 24,000 polypeptide chain. This data conflicts with the previous estimates of M_r 50,000 obtained by Sephadex chromatography of GAT-TsF in extracts (9) and in the crude supernate of C4.4 (12). A number of factors may have contributed to these differing estimates. Purified GAT-TsF behaves as a highly hydrophobic molecule (eluting in 40% propanol from the RP-8 column) and, in its crude form, may interact with a variety of other molecules including lipids. Its hydrophobic behavior also suggests a possible interaction or association with the cell membrane. Alternatively, it is possible that the isolation procedure has caused a fragmentation of a larger molecule from which we have isolated the bioactive fragment. Arguing against this possibility are the data we have obtained with partially purified mRNA isolated from C4.4, which has been used as a template in a cell-free reticulocyte lysate system (unpublished data). The cell-free translated products of C4.4 mRNA have specific suppressor activity. Studies using reverse-phase chromatography, affinity chromatography, and polyacrylamide gel electrophoresis show that the suppressor product in this system has similar chemical and serological properties compared to the TsF isolated from whole cells; moreover, its M_r by polyacrylamide gel electrophoresis is $\approx$19,000, which is very similar to the TsF we have isolated. These data strongly suggest that the M_r of the native suppressor is, in fact, 24,000. The additional M_r can be accounted for by the presence of carbohydrate.

Studies to be reported elsewhere (unpublished data) have demonstrated that pure GAT-TsF has the same serological properties as the crude GAT-TsF (12), suggesting that both the antigen-binding site, the crossreacting idiotype, and the major histocompatibility complex-encoded determinant are present on the M_r 24,000 molecule. Other experiments indicate that this monoclonal TsF inhibits immune responses by the induction of a second set of suppressor T cells (unpublished data).

It is noteworthy that the C4.4 TsF product contains all of the biological and serological properties necessary for its function on a M_r 24,000 polypeptide. Other workers have reported on the biochemical and serological properties of suppressor factors that were either larger multimeric molecules (22, 23) or larger single polypeptides (24, 25)—with M_rs 70,000–300,000. Undoubtedly, some of these factors perform different functions than GAT-TsF. Our experiments and those from other laboratories suggest that the suppressor pathway involves interactions among T cells (26, 27) and results in the production of two or more soluble mediators (reviewed in ref. 28). These new families of antigen-specific proteins should be studied further in terms of both their chemical structure and biological function.

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